

PROTEIN FEEDING ATTENUATES ATTRACTION OF MEXICAN FRUIT FLIES (DIPTERA: TEPHRITIDAE) TO VOLATILE BACTERIAL METABOLITES

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ABSTRACT

We tested the hypothesis that Mexican fruit flies [*Anastrepha ludens* (Loew)] are attracted to odor of tryptic soy broth cultures of *Staphylococcus aureus* (Rosenbach) because they are hungry for protein. First, we demonstrated that attraction to the odor was attenuated by feeding on a relatively complete diet containing sugar, protein, fats, vitamins, and minerals compared to feeding on sugar only; second, we showed that feeding on a diet of casein hydrolysate and sugar in which the percentage of protein was equal to that in the complete diet attenuated attraction to the same degree as the complete diet; and third, we showed that attraction to bacterial odor decreased as percentage of protein increased in a diet containing casein hydrolysate and sugar. Results of the three experiments support the hypothesis that flies are attracted to odor of *S. aureus* cultures largely to find protein. Dietary vitamins, minerals, fats, and percentage of protein as amino acids had no effect.

Key Words: *Anastrepha ludens*, kairomones, bacteria, specific-hunger

RESUMEN

Se puso a prueba la hipótesis de que la mosca Mexicana de la fruta [*Anastrepha ludens* (Loew)] es atraída por el olor de cultivos en caldo de soya de la bacteria *Staphylococcus aureus* por estar ávida de proteína. Primeramente, se comprobó que la atracción de las moscas hacia el olor fue mas tenue cuando estas se alimentaron de una dieta relativamente completa (azúcar, proteína, aceite, vitaminas y minerales) que cuando se alimentaron de una dieta que contenía solamente azúcar; en segundo lugar, se comprobó que alimentándose de una dieta que contenía caseína hidrolizada y azúcar (cantidad de proteína equivalente a la dieta relativamente completa) la respuesta de las moscas fue tenue y del mismo grado que cuando se alimentaron de la dieta relativamente completa; en tercer lugar, se comprobó que la atracción de las moscas hacia el olor de la bacteria disminuyó con el incremento de caseína hidrolizada en la dieta. Los resultados de los tres experimentos apoyan la hipótesis de que las moscas son atraídas por el olor de los cultivos de *S. aureus* porque estas buscan proteína para alimentarse. Los compuestos nutritivos de vitaminas, minerales, aceites, y porcentaje de proteína, en forma de amino ácidos, no afectaron las respuestas.

Odors produced by numerous species of bacteria have now been shown to be attractive to adults of various species of Tephritidae (Drew et al. 1983, Courtice & Drew 1984, Drew & Lloyd 1989, Jang & Nishijima 1990, MacCollom et al. 1992). The Mexican fruit fly [*Anastrepha ludens* (Loew)] is strongly attracted to odors produced during fermentation of culturing media by bacteria from at least four families (Robacker et al. 1991, Martinez et al. 1994). Robacker et al. (1993) later presented evidence that the attractive chemicals, hereafter referred to as bacterial odor, are probably volatile metabolites of the bacterial fermentation process.

The reason for the attractiveness of bacterial odor has been studied in recent work with the Mexican fruit fly but remains unresolved. Robacker (1991) showed that flies fed yeast hydrolysate and sugar were much less responsive to odor of cultures of the bacterium *Staphylococcus aureus* (Rosenbach) than flies fed only sugar and concluded that bacterial odor attracted flies hungry for protein, which is present in yeast hydrolysate. However, Robacker & Garcia (1993) later found that flies fed yeast hydrolysate up until the time of bioassays nevertheless were strongly attracted to bacterial odor. They also showed that sugar deprivation greatly depressed attraction of flies to the odor. This raised the question of whether decreased attraction to bacterial odor in tests where flies had been fed yeast hydrolysate may have been at least partly due to insufficient sugar in the yeast hydrolysate/sugar diets. The role of protein hunger in attraction to bacterial odor was again open for debate. During scrutiny of earlier data, another point of uncertainty came up regarding the composition of yeast hydrolysate itself. As yeast hydrolysate contains fats, vitamins and minerals in addition to protein, we now had to ask if the effects of feeding flies yeast hydrolysate on reducing attraction to bacterial odor may have been due to some nutrient or nutrients other than protein. This seemed like a strong possibility in light of recent work showing that two species of predatory mites fed a diet deficient in carotenoid vitamins were attracted to kairomones produced by prey that contain the carotenoids, while the same two mite species fed diets containing carotenoids did not respond to those kairomones (Dicke et al. 1986, Dicke 1988).

The purpose of this work was to determine how feeding by adult Mexican fruit flies on diets containing various nutrients affected attraction of the flies to bacterial odor produced by action of *S. aureus* strain RGM-1 (Robacker et al. 1991) on tryptic soy broth media. This was done in three experiments. First, a comparison was made of attraction of flies fed a diet containing a relatively complete mixture of nutrients vs only sugar to verify that some nutrient or nutrients in the complete diet would in fact attenuate responses of the flies to the bacterial odor. Next, a comparison was made of attraction of flies fed the complete diet vs a diet containing an equal amount of protein, but no other nutrients (except sucrose), to determine the role of nutrients other than protein. Finally, we tested for effects of diets containing various percentages of protein and sugar and no other nutrients.

MATERIALS AND METHODS

Flies were from a colony maintained for approximately 400 generations with no wild-fly introductions. Mixed-sex groups of 180-200 flies were held in bioassay cages from eclosion with water and various test diets that will be described below. To ensure that flies would not respond strongly to water in both the treatments and the controls, water was provided to them in a light spray during the morning at least one h before bioassays began. Laboratory conditions, both for holding flies and conducting experiments, were $22 \pm 2^\circ\text{C}$ (range), $55 \pm 15\%$ RH (range) and a photoperiod of 13:11 (L:D). Laboratory lighting was a combination of fluorescent and natural light through glass windows.

The bacterial attractant was produced by fermentation of the bacterial strain RGM-1 previously identified as a probable new strain of *S. aureus* from the mouth-parts of a female laboratory-strain Mexican fruit fly (Robacker et al. 1991). While this bacterium probably was introduced into the fruit fly culture from human contact, its cultures are nevertheless very attractive to adult Mexican fruit flies.

RGM-1 was cultured in tryptic soy broth (DIFCO Laboratories, Detroit, MI) in a shaker for 144 h at 30°C . Bacterial culture was centrifuged at 10,000 rpm for 20 min.

The resulting supernatant contained highly attractive material that was used as the attractant source in this research. Previous research had demonstrated that the attractant material in the supernatant was neither bacterial cells nor the tryptic soy broth itself (Robacker et al. 1993). Rather, the attractiveness probably was due to odorant chemicals produced by the bacteria during metabolism of nutrients in the tryptic soy broth.

Bioassays were conducted in $0.3 \times 0.3 \times 0.3$ m, aluminum-framed, aluminum-screened cages. Cage-top bioassays (Robacker et al. 1991) were used in all experiments because this system has provided rapid, quantitative evaluation of attractants ranging from slightly to very attractive. Briefly, the bioassay consisted of placing two filter paper triangles (three cm per side) containing 10 μ l of supernatant of bacterial culture and two papers containing 10 μ l of water, each on one corner on the top of a bioassay cage, and counting the flies beneath the papers 10 times at one minute intervals. Filter papers were raised 0.5 cm above the cage top to ensure that olfaction was solely responsible for attraction of the flies to the filter papers. Bioassays were conducted using 7- to 11-day-old flies. Flies were used for one bioassay, then discarded.

Three experiments were conducted. The purpose of Experiment 1 was to test the hypothesis that flies fed a diet presumed to be more or less nutritionally complete would be less responsive to bacterial odor than flies fed sugar only, as was suggested by results of earlier research (Robacker 1991, Robacker & Garcia 1993). In Experiment 1, two diet types were tested. Ten cages of flies were set up with sugar and water only. Sugar was provided as four sucrose "dainty cubes"[®] (Imperial Sugar Co., Sugarland, TX) placed in petri dishes inside the cages. Water was provided in a plastic vial with a cotton wick inside cages. A second ten cages were prepared with water and a relatively "complete" diet. Water was provided in plastic vials as above and the complete diet was provided in a plastic petri dish. The complete diet was a dry powder mixture containing 20% enzymatic yeast hydrolysate, 20% torula dried yeast, 4% casein, 2% Vanderzant's vitamin fortification mixture for insects, 0.05% cholesterol, 52.35% sucrose (all obtained from U.S. Biochemical Corp., Cleveland, OH), and 1.6% Beck's salts (BIO-SERV, Inc., Frenchtown, N.J.). Both enzymatic yeast hydrolysate and torula dried yeast contained about 50% protein while casein was about 97% protein, according to the manufacturers. Thus the total protein in the complete diet was about 24%. Free amino acids were less than 10% of the diet. The other 50% of yeast hydrolysate and torula dried yeast consisted of unspecified carbohydrates, ash, water, and fiber according to information provided by U.S. Biochemical Corp., and probably small percentages of fats, minerals, and vitamins (Long 1961). Finally, additional sucrose was provided as two sugar cubes (Imperial Sugar Co.) located in the petri dish with the complete diet. The reason for additional sugar was to allow flies to "self-select" the amounts of sugar and protein in their diet (Waldbauer & Friedman 1991). Flies were fed these diets from eclosion and diets were not removed from cages when bioassays were conducted. Experimental procedure was to test two cages, one each of the two diet types, side by side (one m apart) at the same time. Five cages of each diet type were set up and tested as one set. The experiment was repeated with a second set of five cages of each diet set up and tested two weeks later.

Experiment 2 was conducted to determine if nutrients other than protein affect attraction of the flies to bacterial odor. Cages of flies were again set up with one of two diet types. Twenty cages were prepared with the complete diet, two additional sugar cubes and water vials as in Experiment 1. Another 20 cages were prepared with a casein hydrolysate and sugar diet, two additional sugar cubes and water vials. The casein hydrolysate diet was a dry powder mixture containing 27.4% vitamin- and

salt-free casein hydrolysate (ICN Biomedicals, Inc., Irvine, CA) and 72.6% sucrose (U.S. Biochemical Corp.). The casein hydrolysate was about 87.5% protein with little or no other nutrients, according to information provided by ICN Biomedicals. The total protein in the diet was 24%, the same as in the complete diet. Free amino acids were about 18.5% of the diet. Experimental procedure was the same as in Experiment 1. Again, five cages of each diet type were set up and tested as one set. Four sets were tested at two week intervals. Also, two cages fed only four sugar cubes as in Experiment 1 were prepared and tested with each set to verify that low attraction of flies fed the two test diets was not due to fly batch.

Experiment 3 was conducted to determine the relationship between percentage of protein in the diet and attraction of the flies to bacterial odor. Each replication of the experiment consisted of eight cages set up with dry powder diets containing 0, 1, 2, 4, 8, 16, 32, or 64% protein. Casein hydrolysate (ICN Biomedicals, Inc.) was the protein source. Sucrose (U.S. Biochemical Corp.) made up the remainder of the diets. No additional sugar cubes were provided. Water was again provided in plastic vials. Experimental procedure was to set up the eight cages as described above using flies from the same batch and to test them within two h on the same day. The experiment was repeated eight times, each about two weeks apart.

Experiments 1 & 2 were analyzed by paired *t* tests of cages paired by test time (Snedecor & Cochran 1967). Data used in these *t* tests were differences between total counts at bacterial odor and water from each bioassay. Experiment 3 was subjected to 2-way analysis of variance of differences between counts at bacterial odor and water, separating out effects of test day and percentage of protein (Snedecor & Cochran 1967). Effect of percentage of protein was partitioned into linear regression of attraction on percentage of protein on the log₂ scale. Paired *t* tests were also used to compare counts at bacterial odor to counts at water in some cases. Although count differences were used for statistical analyses, means and standard errors (SE) shown in figures were calculated using response ratios from individual bioassays because these were more appropriate for presentation. The response ratio from an individual bioassay was defined as the ratio of the total count at bacterial odor in that bioassay to the mean of total counts at all water-controls in the experiment that included that bioassay.

RESULTS AND DISCUSSION

The results of Experiment 1 are shown in Fig. 1. Flies fed the complete diet were much less responsive to bacterial odor than were flies fed sugar only ($t = 10.4$, $df = 9$, $P < 0.001$). We interpret this to mean that flies fed only sugar were strongly attracted to bacterial odor because they associate bacterial odor with the presence of certain required nutrients that were deficient in the sugar diet. Conversely, flies that fed on the complete diet were not as strongly attracted to bacterial odor because the complete diet partially satisfied their hunger for whatever nutrients they associate with bacterial odor.

Despite lower attraction of flies fed the complete diet compared to flies fed sugar only (Fig. 1), bacterial odor was significantly more attractive than water controls for flies fed the complete diet ($t = 8.5$, $df = 9$, $P < 0.001$). Three possible explanations for this result are: 1) not all of the flies' nutritional needs were met by the complete diet compared to what they associate with bacterial odor; 2) the complete diet has everything they need but the attraction response to bacterial odor does not turn off completely unless hunger of flies is completely satiated, a state that may occur only when their crops are completely full; and/or 3) the bacterial odor contains one or more attractive chemicals that are not associated with the hunger response.

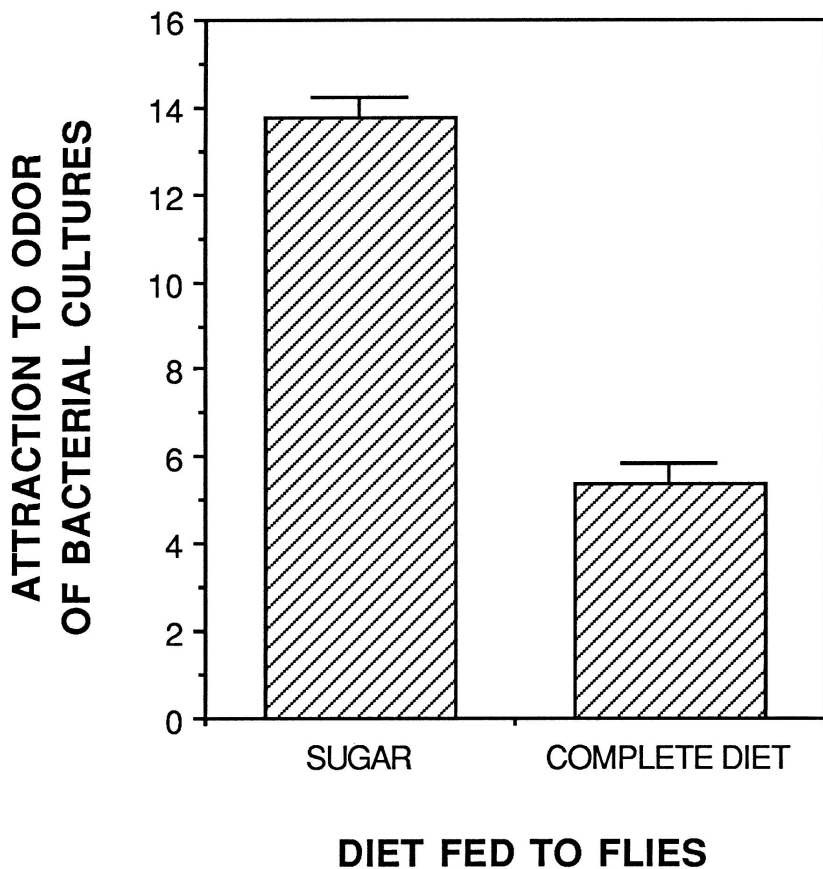
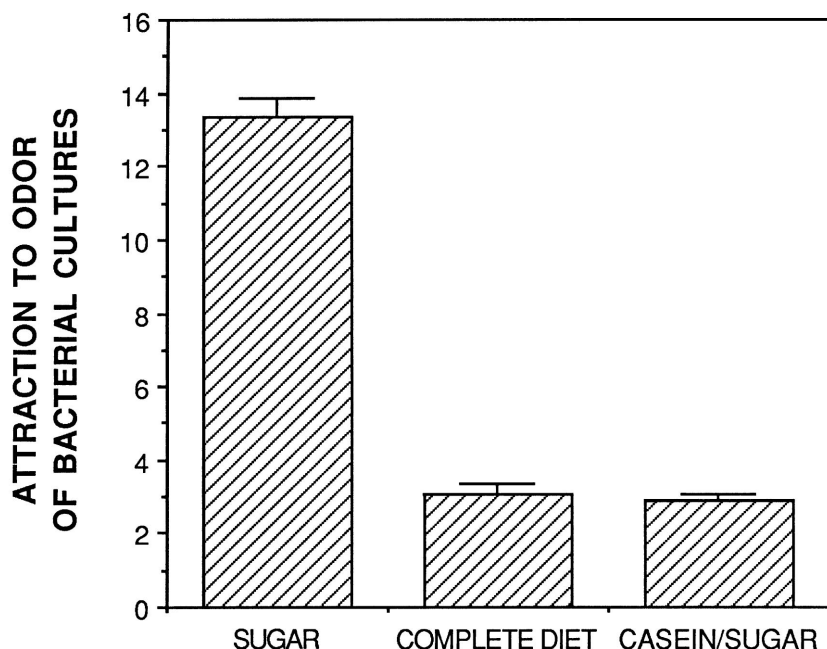


Fig. 1. Attraction to bacterial odor (\pm SE) of Mexican fruit flies fed sugar or a complete diet containing a balance of required nutrients. Bars are response ratios of attraction to bacterial odor relative to attraction to water controls. Attraction of flies fed the two diets was significantly different by a paired t test ($P < 0.001$, $df = 9$).

There was no difference in attraction of flies fed the complete diet or the casein hydrolysate/sugar diet ($t = 1.4$, $df = 19$, $P = 0.2$) (Fig. 2). Note that the two diets were equal in percentage of protein but differed in every other nutrient. For example, the casein hydrolysate diet contained almost no nutrients other than protein and sugar while the complete diet contained protein, sugar, vitamins, minerals, fats, etc. Further, the casein hydrolysate diet contained nearly twice as much of its protein as amino acids as did the complete diet. Indications are that the equal percentage of protein in the two diets was the primary factor determining equal attraction to the bacterial odor. This suggests that flies are attracted to bacterial odor largely because they associate it with the presence of protein.

As in Experiment 1, attraction to bacterial odor of flies fed diets containing protein in Experiment 2 was considerably lower than attraction of flies that were fed sugar only (Fig. 2). Also as in Experiment 1, attraction to bacterial odor nevertheless was



DIET FED TO FLIES

Fig. 2. Attraction to bacterial odor (\pm SE) of Mexican fruit flies fed sugar, a complete diet containing a balance of required nutrients, or a diet containing casein hydrolysate and sugar in which the percentage of protein was the same as that of the complete diet. Bars are response ratios of attraction to bacterial odor relative to attraction to water controls. Attraction of flies fed the complete diet and the casein hydrolysate diet was not significantly different by a paired t test ($P = 0.2$, $df = 19$).

significantly greater than attraction to water controls for flies fed the two protein-containing diets (complete diet: $t = 8.6$, $df = 19$, $P < 0.001$; casein hydrolysate/sugar diet: $t = 8.5$, $df = 19$, $P < 0.001$).

In Experiment 3, attraction of flies to bacterial odor was affected by diet fed to the flies ($F = 44.2$; $df = 6, 42$; $p < 0.001$). Attraction decreased nearly linearly with the \log_e of the percentage of protein ($r^2 = 0.55$, $P < 0.001$) (Fig. 3). Data for 64% protein were not included in Fig. 3 or in the analysis of variance because over 60% of the flies in the cages were dead by the test day, and most of the remaining flies appeared weak. The actual response ratio for the 64% protein diet was 0.6 indicating that fewer flies came to the bacterial odor than to water.

The results of Experiment 3 can be interpreted two ways. One explanation is that attraction of flies to bacterial odor decreased as percentage of protein in the diet increased because protein hunger decreased. This explanation corroborates our conclusion from Experiment 2 that flies are attracted to bacterial odor because they associate it with the presence of protein. However, the percentage of sugar in the diets decreased as the percentage of protein increased so the possibility that diminishing response by the flies may be due to increasing sugar hunger must be considered. This

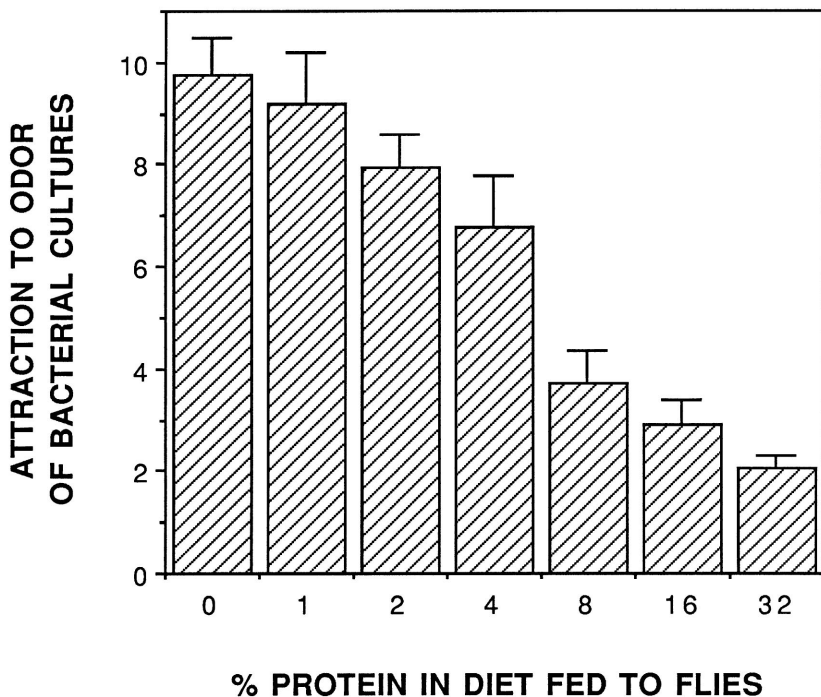


Fig. 3. Attraction to bacterial odor (\pm SE) of Mexican fruit flies fed diets containing sugar and various percentages of casein hydrolysate protein. Bars are response ratios of attraction to bacterial odor relative to attraction to water controls. Attraction of flies decreased nearly linearly with the \log_2 of percentage of protein ($r^2 = 0.55$, $P < 0.001$).

explanation is plausible because Robacker & Garcia (1993) showed that sugar-hunger greatly depresses attraction of Mexican fruit flies to bacterial odor.

We believe the explanation for the results of Experiment 3 is that the decrease in attraction was due to a decrease in protein hunger rather than an increase in sugar-hunger. There are several reasons for this contention. First, the diets depicted in Fig. 3 all contained at least 68% sugar. This percentage is well above the percentage of sugar (52.35%) in the complete diet that was found to optimize Mexican fruit fly fecundity and longevity (D.S.M. unpublished data). Second, most of the effect was manifested before the percentage of sugar in diets had dropped below 92%, a decrease in relative percentage of sugar of only 8% from the 100% sugar diet. At the same time, protein percentage increased from 0 to 8%, a large increase in relative percentage of protein. Thus, the change in sugar percentage probably was insignificant compared to the change in protein. Finally, the results of Experiment 2 in which attraction to bacterial odor were unaffected by a decrease in sugar percentage from 72.6% in the casein hydrolysate/sugar diet to 52.35% in the complete diet suggest that sugar percentage is unimportant as long as it is higher than some undetermined threshold level.

We conclude that attraction of Mexican fruit flies to odor of tryptic soy broth cultures of *S. aureus* strain RGM-1 is primarily due to hunger for protein. Presence or absence of fats, vitamins, and minerals seems unimportant. We suggest this is a

"specific hunger" (Dethier 1976) for protein that translates into appetitive search for protein food sources due to an innate neural association of bacterial odor with the presence of protein. Possibly, attraction of fruit flies to bacteria generally may be governed by protein-hunger, based on the work of Drew & Lloyd (1989) that implicated bacteria as a natural protein source for fruit flies.

ENDNOTE

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REFERENCES CITED

- COURTICE, A. C., AND R. A. I. DREW. 1984. Bacterial regulation of abundance in tropical fruit flies (Diptera: Tephritidae). *Australian Zool.* 21: 251-268.
- DETHIER, V. G. 1976. *The Hungry Fly*. Harvard University Press, Cambridge, Massachusetts.
- DICKE, M., M. W. SABELIS, AND A. GROENEVELD. 1986. Vitamin A deficiency modifies response of predatory mite *Amblyseius potentillae* to volatile kairomone of two-spotted spider mite, *Tetranychus urticae*. *J. Chem. Ecol.* 12: 1389-1396.
- DICKE, M. 1988. Prey preference of the phytoseiid mite *Typhlodromus pyri* 1. Response to volatile kairomones. *Exp. Appl. Acarol.* 4: 1-13.
- DREW, R. A. I., A. C. COURTICE, AND D. S. TEAKLE. 1983. Bacteria as a natural source of food for adult fruit flies (Diptera: Tephritidae). *Oecologia*. 60: 279-284.
- DREW, R. A. I., AND A. C. LLOYD. 1989. Bacteria associated with fruit flies and their host plants, pp. 131-140 in A. S. Robinson and G. Hooper [eds.], *Fruit Flies: Their Biology, Natural Enemies and Control*. Vol. 3A. Elsevier, Amsterdam.
- JANG, E. B., AND K. A. NISHIJIMA. 1990. Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). *Environ. Entomol.* 19: 1726-1731.
- LONG, C. 1961. *Biochemists' Handbook*. Van Nostrand, Princeton, New Jersey.
- MACCOLLOM, G. B., C. R. LAUZON, R. W. WEIRES, JR., AND A. A. RUTKOWSKI. 1992. Attraction of adult apple maggot (Diptera: Tephritidae) to microbial isolates. *J. Econ. Entomol.* 85: 83-87.
- MARTINEZ, A. J., D. C. ROBACKER, J. A. GARCIA, AND K. L. ESAU. 1994. Laboratory and field olfactory attraction of the Mexican fruit fly (Diptera: Tephritidae) to metabolites of bacterial species. *Florida Entomol.* 77: 117-126.
- ROBACKER, D. C. 1991. Specific hunger in *Anastrepha ludens* (Diptera: Tephritidae): Effects on attractiveness of proteinaceous and fruit-derived lures. *Environ. Entomol.* 20: 1680-1686.
- ROBACKER, D. C., J. A. GARCIA, A. J. MARTINEZ, AND M. G. KAUFMAN. 1991. Strain of *Staphylococcus* attractive to laboratory strain *Anastrepha ludens* (Diptera: Tephritidae). *Ann. Entomol. Soc. America*. 84: 555-559.
- ROBACKER, D. C. AND J. A. GARCIA. 1993. Effects of age, time of day, feeding history, and gamma irradiation on attraction of Mexican fruit flies (Diptera: Tephritidae), to bacterial odor in laboratory experiments. *Environ. Entomol.* 22: 1367-1374.
- ROBACKER, D. C., W. C. WARFIELD, AND R. F. ALBACH. 1993. Partial characterization and HPLC isolation of bacteria-produced attractants for the Mexican fruit fly, *Anastrepha ludens*. *J. Chem. Ecol.* 19: 543-557.
- SNEDECOR, G. W., AND W. G. COCHRAN. 1967. *Statistical Methods*. The Iowa State University Press, Ames, Iowa.
- WALDBAUER, G. P., AND S. FRIEDMAN. 1991. Self-selection of optimal diets by insects. *Annu. Rev. Entomol.* 36: 43-63.